Bio-control trials of *Chaetomium spirale* ND35 against apple canker

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Abstract: A new endophytic antagonistic fungus, *Chaetomium spirale* ND35 from *Populus tomentosa, was* reported. The bio-control trials of *C. spirale* ND35 against the *Valsa* Canker of apple were preliminarily investigated. The results of dual culture on PDA plate showed that C. *spirale* ND35 was capable of strong antagonism against *Valsa ceratosperma*, and for inhibiting the mycelial growth of *V. ceratosperma*,, the crude extract of liquid culture of corn steep powder broth was more effective than that one of malt extract broth (MEB). The results of bio-control in greenhouse and field indicated that the disease incidence of apple tree treated with *C. spirale* ND35 was lower significantly than that treated by other methods. The re-isolation experiment suggested that *C. spirale* ND35 could colonize in stems and branches of apple trees successfully, and the ND35 colonization rate of the treatment with solid wheat bran culture was higher than that of corn steep powder broth, but the field experiment result the control effect of liquid culture of *C. spirale* ND35 was better than that of solid culture.

Keywords: *Chaetomium spirale;* Apple tree canker; Antagonism; Bio-control; *Valsa ceratosperma*

CLC number: S436.611.11; S476 **Document code:** A **Article ID**: 1007-662X(2005)02-0121-04

Introduction

The apple-tree canker caused by *Valsa ceratosperma* brings serious barriers in apple production (Chen 1980). In China, traditional chemical fungicides currently used to control this disease also kill some beneficial microbes and pollute, environment as well (Xiang *et al.* 1992), at the same time, the pathogens develop resistance to the fungicides increasingly. Thus, much attention has been drown on bio-control that could be used as a supplied form or substitute for chemical.

Chaetomium Kunze ex Fr., widely distributed in nature, is one of the major genera of ascomycetes and more than 300 *Chaetomium* species have been described (Reissinger *et al.* 2003). It has long history of ascospore and strong capacity of resistance to dry and lower temperature and other environment. Liu *et al* (1999) reported that the ascospores kept in the air around two and half years still had a high germination capacity (90%). *Chaetomium* could be regarded as a kind of ideal fungus for bio-control. It has demonstrated antagonism against many pathogens, such as *Rhizoctonia solani, Pythium ultimum,* which cause dampling off, as well as *Venturia inaequalis* which cause the apple scab (Cullen *et al.* 1984 ; Di Pietro *et al.* 1992). However, controlling forest diseases by *Chaetomium,* has been rarely reported in China. The objective of this study is to provide solid foundation for applying *Chaetomium* in control of apple Valsa canker.

Materials and methods

Fungi and plants

A strain ND35 of *Chaetomium spirale*, an endophytic fungus, was isolated from *Populus tomentosa*. Strain M of *Valsa ceratosperma* was isolated from canker on shoots and stems of apple tree. Two-year-old inoculated shoots of apple tree (*Malus pumila* Mill. cv. Red Star) were collected from Guanghuasi Forest Farm in Mountain Culai of Tai'an City, Shandong Province, China.

Dual culture experiment

A dual-culture technique (Gao 2002) was modified and used to study antagonism. Culture discs (5 mm in diameter) of *C. spirale* ND35 and of *V. ceratosperma* were taken from the edge of an actively growing colonies and transferred to 90-mm PDA culture plates on opposite sides. The distance between the inoculation points of pathogen and the potential antagonist was 4 cm. The discs of pathogen on PDA alone served as control. Four replicated plates were inoculated at 25 ℃, lasted for 10 days, and the colonies radius of pathogen growth related to growth in the absence of the potential control agent was measured every day after inoculation. Growth inhibitory rate for pathogen (%) is computed by the following equation:

$$G_{IR} = (R_c - R_{dc})/R_c \times 100$$

where, G_{IR} is Growth inhibitory rate for pathogen (%), R_c is Pathogenic colony radius of control, and R_{dc} is Pathogenic colony radius in dual culture

Inhibitory effect of different crud extract of culture

Liquid culture media for growth are as follows; 3% Corn Steep Power Broth; 1% Malt Extract Broth. Flasks containing 100 ml liquid medium were inoculated with a 5-mm disc cut from the margin of a vigorously growing culture of *C. spirale* ND35. The flasks were incubated in a rotary shaker at 130 rpm for 15 days at 25 ℃. Cultures were filtered through two layers of filter paper, and the filtrates were sterilized for 3 times by bacterial filtration.

An agar plug sourced from the 2–day-old culture of *V. ceratosperma* was placed in the center of each plate containing 15 ml PDA. 4 pores (pore size 5 mm) were made at equal distance to center on the PDA plate with punch. Subsequently two filtrates were added in two opposite pores, sterile distilled water (SDW) as control was added in another two opposite pores. The plates

Foundaition item: This research was supported by National Natural Science Foundation of China (Grant No. 30100143)

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Received date: 2005-03-05

Responsible editor: Chai Ruihai

were incubated continually for 4-5 days, and efficacy was observed.

Testing of artificial inoculation

The method of Gao (2000) was used for testing artificial inoculation. Two-year-old shoots of apple tree were cut into 25-cm segments in length after washed with tap water, drying, and the upper parts of segments were sealed with paraffin wax. The 1/3 and 2/3 parts of shoots were scalded with a heated scalpel until the bark became dark accordingly. The antagonist and pathogen were inoculated with a 5-mm agar disc containing mycelia. *C. spirale* was inoculated one week before the pathogen (ND35-M).

Controls were set in three ways: 1) Wounded shoots were inoculated only with the pathogen (C1); 2) Apple shoots were just scalded with heated scalpel, but not inoculated (C2); 3) Healthy shoots without being wounded (H). Three replicates were made for each treatment and 5 shoots were used for each replicate. After inoculation, the wounds were bound with sterilized de-fatted cotton and plastic membrane and kept moist with SDW. The inoculated apple shoots were incubated at 25℃ in vessels containing tap water that was replaced daily. Ten days after inoculation, the de-fatted cotton and plastic membrane were removed, and the inoculated shoots were examined for symptoms during the next 32 days. The standard of judgment was used: Around scalded wound on shoots, whether disease scars and size present or not, or fruit bodies of pathogen produce or not. The number of infected shoots and disease scar were recorded and disease incidence of inoculated shoots was calculated.

Re-isolation from inoculated apple shoots

The method of Xiang (1991) was used to determine the survival of antagonists and their antagonistic effectiveness on shoots. Either infected or non-infected bark samples were taken from inoculated shoots of each treatment. The bark samples were cut into 0.5 cm ×0.5 cm pieces, wrapped in muslin and placed in 70% ethanol for 30 s. After several rinses in SDW, the specimens were placed in 0.1% mercuric chloride for 3 min. Five pieces were put on a prepared plate containing 15 ml PDA after rinsing twice with SDW. There were 30 specimens for each treatment. Plates were incubated at 25℃ for 7 days. After growth of mycelia from the bark samples on plates, the percentage of re-isolation colonies was recorded.

Production of liquid fermentation of *C. spirale* ND35

Maize powder of 60 g was put in 1-L water, mixed uniformly, and cooked for 1 h. after then, the liquid meida were filled in flasks. Each flask with 50 ml culture liquid was autoclaved at 121 ℃ for 30 min. Six agar discs (1 cm in diameter) containing actively growing mycelia of *C. spirale* were inoculated in each flask and incubated in a rotary shaker at 120 rpm for 10 days at 25℃.

Preparation of wheat bran inoculum of *C. spirale* ND35

Wheat bran inoculum of *C. spirale* was prepared as follows: 50-g wheat bran and 130-mL water were mixed in autoclavable plastic bags and autoclaved for 30 min. The bran was inoculated with an ascopore suspension of *C. spirale* (10^6 spores per bag). The bran was mixed up, and the closed bag was incubated at 25℃ for 7 days.

Testing of bio-control in the field

On March 18 and April 14, 2002, bio-control trials of C. spirale ND35 against apple-tree canker was conducted twice at an orchard with serious canker on 10-year-old apple trees in Guanghuasi Forest Farm in Mountain Culai of Tai'an City, Shandong Province, China.

The infected parts of barks on stems and branches were cut. After scab barks of infected apple trees were wholly cut, the liquid cultures and solid preparation were inoculated on the wound respectively. The treatments are as follows: wheat bran inoculum, liquid fermentation of corn steep powder broth, and water control.

The experiment was carried out by 3 replications, and three trees were randomly selected for each replication. After inoculation, the wound were wrapped with plastic membrane to proof fungicide, and the plastic membrane was removed at the beginning of May. Antagonist and pathogen were isolated and the formation of healed issue was inspected by taken causal samples on May 30. The isolation percentage of pathogen and antagonist were calculated.

Results

Dual culture experiment

The experimental result indicates that *C. spirale* ND35 has strong antagonism against *V. ceratosperma* and the percentage of growth inhibition of pathogen was 78.46. The inhibition zone in the interaction area of the two colonies of *C. spirale* ND35 and *V. ceratosperma* was observed. The hyphae of *V. ceratosperma* in the edge of inhibition zone formed brawn tubercle and became deformity (Table 1).

Table 1. The inhibition percentage of pathogenic growth

Treatment	Colony radius of pathogen (mm)			Average (mm)	Inhibition percentage*(%)
M+ND35	13.0	14.0	15.0	14.0	78.46
Control	65.0	65.0	65.0	65.0	---

*Inhibition percentage = (Colony radius of control - Colony radius of pathogen) / Colony radius of control

Effect of artificial inoculation of apple shoots

The inoculation test showed that the incidence of infected apple shoots was reduced 77.5% after 32 days pre-inoculation with *C. spirale* ND35, indicating that *C. spirale* ND35 had significant antagonism against *V. ceratosperma* on shoots. The incidence of non-inoculated shoots with wound was higher than that of healthy shoots. This demonstrated that apple shoots decreased resistance to pathogen because of the wound and this condition was advantageous to the development of *V. ceratosperma* within shoots (Table 2).

Table 2. The incidence of artificial inoculation of apple shoots

Treatments	Incidence (%)		
	Replication 1	Replication 2	Replication 3
C1	90.0	90.0	90.0
ND35-M	12.5	12.5	12.5
C2	10.0	10.0	10.0
H	0	0	0

Note: C1: Inoculation with only pathogen; ND35-M: Inoculation with *C. spirale* before pathogen for 1W; C2: Wounded shoots without inoculation; H: Healthy shoots without inoculation

Re-isolation of inoculated apple shoots

Results in Table 3 revealed that re-isolation of the antagonists from inoculated apple shoots showed that the appearance of *C. spirale* ND35 significantly differed among various inoculation treatments (ND35-M, C1, C2, H). The re-isolation percentage of *C. spirale* ND35 in the treatment ND35-M (pre-inoculation for one week before the pathogen) was 31.1, apparently higher than that on the other treatments. This can be explained that *C. spirale* ND35 colonized easily in shoots. Moreover re-isolation percentage of pathogen in the treatment C1 (Inoculation only with the pathogen) was 84.4, which was greater than that of the treatment ND35-M. Meanwhile obviously inhibition against colonization and spread of *V. ceratosperma* in shoots could be demonstrated by *C. spirale* ND35 as well.

Table 3. Re-isolation percentage of artificial inoculation of apple shoots

Treatment	*C. spirale* ND35			*V. ceratosperma*		
	Average (%)	Significant difference		Average (%)	Significant difference	
		P=0.05	P=0.01		P=0.05	P=0.01
C1	2.2	b	B	84.4	a	A
ND35-M	31.1	a	A	1.1	b	B
C2	10	b	AB	0	b	B
H	10	B	AB	0	b	B

Dissimilar letters are significantly different (p=0.05, p=0.01)

Inhibitory effect of the crude extract of culture

The crude extract of culture in corn steep powder broth was able to inhibit the mycelial growth of *V. ceratosperma* more effectively compared with that one in malt extract broth. It demonstrated that the kinds and ingredient of medium influenced greatly production of inhibitory compounds of *C. spirale* ND35. (data not shown)

Field trial of *C. spirale* ND35 against apple canker

The liquid culture was more advantageous to formation of healed tissue of apple tree than solid culture, and the control effect of liquid culture was apparently better than that of solid culture (Table. 4).

Table 4. Control effect of *C. spirale* ND35 in the field

Treatments	Formation of healed tissue	Percentage of re-occurrence (%)	Heal percentage (%)	Control effect (%)
Solid culture	++	22.2	77.8	50.0
Liquid culture	+++	9.1	90.9	79.5
Control	+	44.4	55.6	--

* +: The formation of healed tissue was not good, plant tended towards death; ++: The formation of healed tissue was good, a few wounds formed some wider healed tissue; +++: The formation of healed tissue was very good, many wounds formed some wider healed tissue.

According to the previous study (Di Pietro, 1992), the liquid culture i. e the fermentation of corn steep powder has more advantageous to production of Chaetomiumin. Chaetomiumin could kill pathogen in plant directly. Moreover *C. spirale* ND35 of solid culture needs to colonize and expand in plant and to accumulate adequate population number for suppressing and restraining further spread of pathogen. This is the reason that the control effect of solid culture was not as good as that of liquid culture. In addition, solid culture was wrapped with plastic membrane to keep moist, consequently, much water was not advantageous to heal of wound, and some nutrients of solid culture might promote growth of pathogen.

Isolation of *C. spirale* ND35 and *V. ceratosperma* in the field

Isolation percentage of *C. spirale* ND35 of solid culture (78.3%) was higher than that of liquid culture (37.8%). The isolation rate of pathogen in treatment of solid and liquid cultures was equaled to zero, indicating better control effects.

Table 5. The isolation percentage of *C. spirale* ND35 and *V. ceratosperma* in the field

Treatments	Isolation percentage(%)	
	C. spirale ND35	*V. ceratosperma*
Solid culture	78.3	***0***
Liquid culture	37.8	*0*
control	5.0	*6.7*

Discussions

In the past several decades, one of the most common, *C. globosum* Kunze ex Fr. has received considerable attention as a potential biocontrol agent for a number of soil and air-borne plant pathogens, including *R. solani, Pythium ultimum, Sclerotinia sclerotiorum* and *Venturia inaequalis* Etc. The mechanisms of fungi suppression by *C. globosum* may be mainly based on antibiosis. In addition, mycoparasitism judged by hyphal coiling of the antagonist around *R. solani* and *Alternalia brassicicola* in dual cultures was observed (Andrews *et al.* 1983; Cullen *et al.* 1984; Vannaeci *et al.* 1987; Walther *et al.* 1988; Di Pietro *et al.* 1992; Knudsen *et al.* 1995; Pereira *et al.* 1997; Monaco *et al.* 1998).

In this study, a new endophytic antagonistic fungus, *C. spirale* ND35 from *Populus tomentosa was* reported. It has ability of suppression of *Valsa* canker of apple both *in vtro* and *in vivo*. The bio-control trials under greenhouse and field conditions proved that *C. spirale* ND35 is a kind of effective bio-control agent against apple canker, as well as it could colonize stems and branches of apple tree successfully. The crude extracts from both liquid and solid cultures of *C. spirale* ND35 could inhibit mycelial growth of *V. ceratosperma* effectively, implying that antibiotic substances might be involved in antifungal activity of *C. spirale* ND35. On the contrary, Cell wall degrading enzymes (CWDEs) responsible for mycoparasitism of *C. spirale* ND35 could be another mechanism of biocontrol (Gao *et al.* 2005), but the synergistically antifungal interaction of between antibiotics and CWDEs need further investigation in order to better understand the modes of actions against phytopathogenic fungi and to improve bio-control activity by C. spirale ND35 in field

Acknowledgement

The authors thank Dr. Metzler B. for identification of *Chaetomium spirale* ND35.

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